



Identification and Characterization of Major Flavonoids in Extracts from an Unexplored Medicinal Herb *Orostachys fimbriata*

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Abstract: *Orostachys* species have been recognized as medicinal herbs in East Asia. Although *O. fimbriata* is known as a traditional medicine, its antioxidant properties have not been investigated compared to other *Orostachys* species. In this study, we characterized the antioxidant compounds and determined the antioxidant activity of *O. fimbriata* for the first time. As a result, 1 g of *O. fimbriata* extracts contains 288.5 ± 7.4 mg polyphenols, which contains 159.7 ± 8.3 mg flavonoids. In particular, 21.6%, 6.6%, and 2.6% of the total flavonoids were identified as epicatechin gallate, quercetin, and kaempferol, respectively, by LC-MS system. The DPPH IC₅₀, ABTS IC₅₀, and FRAP value of the extracts was determined to be 27.6 ± 5.5 µg/mL, 125.7 ± 6.0 µg/mL, and 115.0 ± 4.4 mmol/L, respectively. These activities were 30–57% of the positive control, ascorbic acid. In conclusion, it was demonstrated that *O. fimbriata* has outstanding antioxidant properties. This study highlights the need for further investigations toward in-depth research on the pharmacological functions of *O. fimbriata*.

Keywords: *Orostachys fimbriata*; medicinal herb; antioxidant; flavonoid; epicatechin gallate; quercetin; kaempferol

1. Introduction

Orostachys is a biennial herb belonging to the family Crassulaceae and is widely distributed in East Asia including China, Korea, and Mongolia. *O. japonicus* and *O. fimbriata* have traditionally been used as medicinal herbs [1,2]. The pharmacological effects of these two species, such as anti-inflammation and promoting wound healing, are introduced in the Chinese Pharmacopoeia [2]. Among *Orostachys* spp., *O. japonicus* has been a relatively well-studied species [3,4]. Numerous studies have reported that the *O. japonicus* extracts exhibited various biological activities such as antimicrobial activity [5], immunostimulatory activity [6], anti-cancer activity [4], and antioxidant activity [7]. It is recognized that this is because *Orostachys* extracts contain many bioactive compounds such as flavonoids [8]. Flavonoids are a class of polyphenolic secondary metabolites, and their chemical structure is a 15-carbon skeleton consisting of two phenyl rings and one heterocycle (Figure 1a) [9]. These are strong antioxidants because of their several phenolic hydroxyl groups, which can be easily oxidized [10]. These natural compounds are considered essential products in various industries in medicine and pharmaceutical applications.

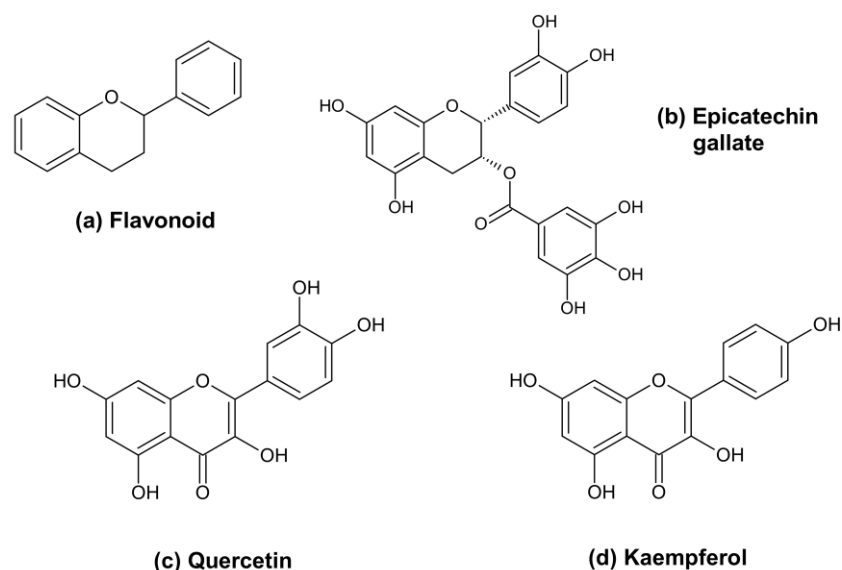


Figure 1. The basic skeleton of flavonoids (a) and structures of major flavonoids in *Orostachys*: epicatechin gallate (b), quercetin (c), and kaempferol (d).

Characterization of antioxidant properties of plant extracts can be performed through the quantification of major antioxidant groups and the measurement of antioxidant activity. In *Orostachys* species, including *O. japonicus* and *O. cartilaginosa*, epicatechin gallate, quercetin, and kaempferol are known to be the major flavonoids (Figure 1) [3,11,12]. According to Lee et al. [3], epicatechin gallate accounted for approximately 39% of the total flavonoids in *O. japonicus* extracts. Although many scientific researches have been conducted on *O. japonicus*, there are few reports on *O. fimbriata*. Moreover, no studies have even been carried out to determine the antioxidant activity of *O. fimbriata* extracts. Fundamental studies are needed to characterize the antioxidant properties of *O. fimbriata*, which have been used as a traditional medicinal plant for over 1000 years in East Asia.

In this study, we aimed to perform the antioxidant characterization of the extracts from an unexplored medicinal herb *O. fimbriata*. The total polyphenol content (TPC) and total flavonoid content (TFC) in the *O. fimbriata* extracts were quantified, and the antioxidant activity of the extracts was evaluated. In addition, the major flavonoids in *O. fimbriata* extracts were identified by LC-DAD-ESI-MS analysis. To the best of our knowledge, there is no literature identifying the major flavonoids of *O. fimbriata*, and therefore this study is the first attempt.

2. Materials and Methods

2.1. Materials

Folin–Ciocalteu reagent, sodium nitrite solution (NaNO_2), gallic acid, rutin, ascorbic acid, sodium carbonate solution (Na_2CO_3), 2,2-Diphenyl-1-picrylhydrazyl radical (DPPH), 2,2'-Azino-bis(3-ethyl-benzothiazoline)-6-sulfonic acid (ABTS), potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$), 2,4,6-Tripyridyl-S-triazine (TPTZ), ferric chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), sodium acetate trihydrate ($\text{CH}_3\text{CO}_2\text{Na} \cdot 3\text{H}_2\text{O}$), and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol and acetonitrile were purchased from Samchun Chemical (Kangnam-gu, Seoul, Republic of Korea). Aluminum chloride (AlCl_3), sodium hydroxide (NaOH), hydrochloric acid (HCl), and formic acid were purchased from Duksan Chemical (Ansan-si, Gyeonggi-do, Republic of Korea). Kaempferol hydrate was purchased from Tokyo chemical industry (Tokyo, Japan). Epicatechin gallate was purchased from Biofron (La Mirada, CA, USA). All reagents used in this study were analytical grade.

2.2. Plant Collection and Voucher Specimen Information

Orostachys fimbriata (Turcz.) A. Berger. (Shown in Figure 2) was collected in Dashichilen district, Bulgan province, Mongolia and identified by Dr. Badamtsetseg Bazarragchaa at National History Museum of Mongolia in August, 2014. A voucher specimen (accession number, KRIB 0054598) of the retained material is preserved at the herbarium of KRIBB.

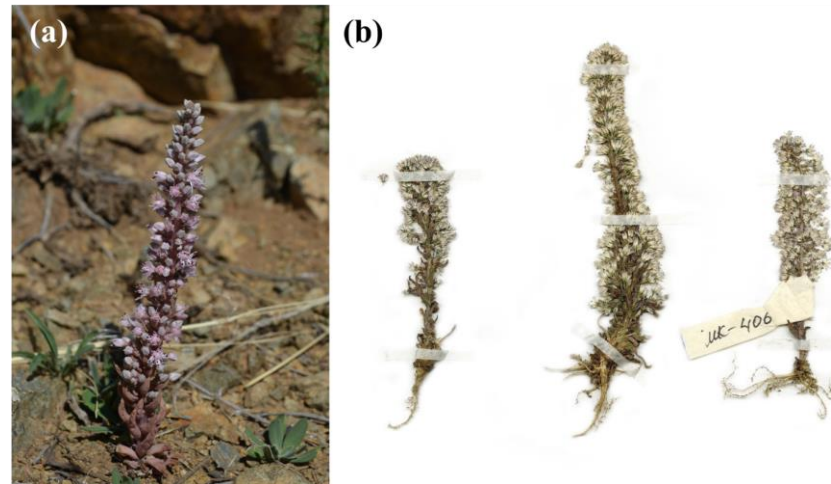


Figure 2. Mongolian herb *Orostachys fimbriata* (Turcz.) A. Berger. (a) and its samples (b).

2.3. Preparation of *Orostachys fimbriata* Extracts

The leaves, shoots, and flowers of *O. fimbriata* (56 g, dry basis) were extracted with 1 L of 99.9% (*v/v*) methanol repeating sonication (15 min) and resting (2 h) for 3 days at 45 °C. The resultant product was filtered with non-fluorescence cottons, and concentrated by rotary evaporator (N-1000SWD, EYELA) under reduced pressure at 45 °C. Finally, total 4.69 g of methanol extract of *O. fimbriata* was obtained by freeze-drying.

2.4. Quantification of Total Polyphenol Content

Total polyphenol content (TPC) of *O. fimbriata* extracts was quantified according to the Folin–Ciocalteu colorimetric method [13]. Briefly, 10 µL of the extracts were mixed with 790 µL of distilled water (DW) and 50 µL of Folin–Ciocalteu reagent. The mixture was incubated at 30 °C for 8 min in a water bath (BHS-2, JOANLAB, Huzhou, China). Then, a volume of 150 µL of 20% Na₂CO₃ solution was added to the mixture and reacted at 25 °C for 1 h. Finally, the absorbance (*A*) of the reaction mixture was measured at 765 nm using an ultraviolet-visible (UV) spectrophotometer (DU[®] 730, Beckman Coulter, Brea, CA, USA). All experiments were performed in triplicate and the results were expressed as the average. TPC was expressed as mg gallic acid equivalent (GAE) per g extract.

2.5. Quantification of Total Flavonoid Content

Total flavonoid content (TFC) of *O. fimbriata* extracts was determined according to aluminium chloride (AlCl₃) colorimetric method with some modification [13]. First, 50 µL extracts were mixed with 30 µL 5% NaNO₂ and reacted at 25 °C for 6 min in the water bath. Next, a volume of 50 µL of 10% AlCl₃ solution was added to the mixture. The mixture was incubated at 25 °C for 5 min. Then, 300 µL of 1 M NaOH and 1 mL of DW were added to the mixture and further incubated at 25 °C for 15 min. Finally, the absorbance (*A*) of the reaction mixture was measured at 510 nm using the UV spectrophotometer. All experiments were performed in triplicate and the results were expressed as the average. TFC was expressed as mg rutin equivalent (RE) per g extract.

2.6. Determination of Antioxidant Activity

To evaluate the antioxidant activity of *O. fimbriata* extracts, DPPH free radical scavenging activity, ABTS cation radical (ABTS^{•+}) scavenging activity, and ferric-reducing antioxidant power (FRAP) were measured according to the reported methods [13–15], respectively. All experiments were carried out in triplicate and the results were expressed as the average. Radical scavenging activity of the extracts was calculated using Equation (1):

$$\text{Radical scavenging activity (\%)} = (1 - A_{\text{sample}}/A_{\text{control}}) \times 100, \quad (1)$$

where A is the absorbance at a specific wavelength against the methanol as the blank solution.

2.6.1. DPPH Assay

A 500 μL of DPPH working solution (0.25 mM DPPH in methanol) was added to 500 μL of the extracts. For the control sample, 500 μL of methanol was used instead of the extracts. The mixture was vortex-mixed and reacted at 25 °C for 30 min in the water bath. After the reaction, the absorbance (A) of the reactant was measured at 517 nm using the UV spectrophotometer. DPPH free radical scavenging activity was calculated using Equation (1) and the results were expressed as IC_{50} ($\mu\text{g}/\text{mL}$), which is the concentration of the extracts required to scavenge 50% of the initial radicals. Ascorbic acid as a standard antioxidant was also tested with the same procedure.

2.6.2. ABTS Assay

To prepare ABTS working solution (ABTS^{•+} solution), a 7 mM ABTS solution was mixed with 2.45 mM $\text{K}_2\text{S}_2\text{O}_8$ (1:1, v/v) and stored at room temperature for 12 h. A volume of 950 μL of the working solution was added to 50 μL of the extracts. For the control sample, 50 μL of methanol was used instead of the extracts. The mixture was vortex-mixed and reacted at 25 °C for 30 min in the water bath. After the reaction, the absorbance (A) of the reactant was measured at 734 nm using the UV spectrophotometer. ABTS^{•+} scavenging activity was calculated using Equation (1) and the results were expressed as IC_{50} ($\mu\text{g}/\text{mL}$). Ascorbic acid as a standard antioxidant was also tested with the same procedure.

2.6.3. FRAP Assay

The FRAP working solution was prepared by mixing 300 mM sodium acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ in a volume ratio of 10:1:1. The working solution was used within 3 h after preparation. A 300 μL of DW was placed in a 1.5 mL e-tube and soaked at 37 °C for 5 min in the water bath. Then, the extracts and the working solution were added to the e-tube by 30 μL and 900 μL , respectively. For the control sample, 30 μL of DW was used instead of the extracts. The mixture was vortex-mixed and stored at 37 °C for 4 min in the water bath. Finally, the absorbance (A) of the reaction mixture was measured at 593 nm using the UV spectrophotometer. For use as a quality control sample, the FRAP value of ascorbic acid was also analyzed by the same procedure. The FRAP values were determined using Equation (2):

$$\text{FRAP value (\mu mol/L)} = (A_{\text{sample}}/A_{\text{control}}) \times \text{Fe}^{2+} \text{ standard concentration.} \quad (2)$$

2.7. Analytical Methods

The flavonoids in *O. fimbriata* extracts were analyzed using high-performance liquid chromatography (HPLC)-diode array detector (DAD) or -DAD-mass spectrometry (MS) system. The following conditions were set for the HPLC-DAD or HPLC-DAD-MS analysis: column, INNO column C18 (5 μm , 4.6 mm \times 250 mm); detector, Agilent 1260 Infinity II DAD HS (G7117C) at 250 nm or 280 nm; column temperature, 25 °C; flow rate, 0.8 mL/min; and sample injection volume, 5 μL .

The gradient elution for the HPLC-DAD analysis was as follows: solvent A, acetonitrile; solvent B, 0.03% (v/v) phosphoric acid in DW; start, 10% A and 90% B; 15 min, 20% A

and 80% B; 28 min, 40% A and 60% B; 36 min, 75% A and 25% B; 38 min, 10% A and 90% B; and 50 min, 10% A and 90% B. The gradient elution for the HPLC-DAD-MS analysis was as follows: solvent A, 0.01% (*v/v*) formic acid in DW; solvent B, 0.01% (*v/v*) formic acid in acetonitrile; start, 90% A and 10% B; 15 min, 80% A and 20% B; 28 min, 60% A and 40% B; 36 min, 25% A and 75% B; 38 min, 90% A and 10% B; and 40 min, 90% A and 10% B. The identification of flavonoids was performed by the SIM mode. MS analysis was carried out under the following conditions: capillary voltage, 4000 V; Drying gas flow, 12 L/min; nebulizer pressure, 35 psi; and drying gas temperature, 350 °C.

3. Results and Discussion

3.1. Investigation of Antioxidant Properties of *Orostachys fimbriata* Extracts

The antioxidant content (TPC and TFC) and antioxidant activity of *O. fimbriata* extracts were analyzed, and the results are shown in Table 1. It was found that 1 g of the extract contained 288.5 ± 7.4 mg of polyphenols. In particular, it was determined that more than half of polyphenols were flavonoids (159.7 ± 8.3 mg/g extract). These results were similar to the report of Jin et al. [16], who demonstrated that TFC (15.8 mg/g extract) in *O. japonicus* accounted for more than 50% of TPC (23.8 mg/g extract).

Table 1. Antioxidant content and antioxidant activity of *Orostachys fimbriata* extracts.

Parameters	Sample	
	<i>O. fimbriata</i> Extracts	Ascorbic Acid
TPC (mg GAE/g extract)	288.5 ± 7.4	-
TFC (mg RE/g extract)	159.7 ± 8.3	-
DPPH IC ₅₀ (µg/mL)	27.6 ± 5.5	9.4 ± 1.9
ABTS IC ₅₀ (µg/mL)	125.7 ± 6.0	71.8 ± 0.2
FRAP value (mmol/L)	115.0 ± 4.4	388.0 ± 16.7

TPC, total polyphenol content; TFC, total flavonoid content.

Flavonoids have been recognized as significant contributors to antioxidant activity due to their unique redox properties [17]. Scavenging activity assays such as DPPH and ABTS assays are based on the ability to scavenge stable free radicals. Reducing power assays, including FRAP assay, are based on the ability to accept electrons from transition metals [18,19]. As each method allows the detection of a different antioxidant group, analysis based on multiple standard methods is required [20]. We evaluated the antioxidant activity of *O. fimbriata* extracts using DPPH, ABTS, and FRAP assays. As a result, DPPH IC₅₀ and ABTS IC₅₀ value of the extracts was determined to be 27.6 ± 5.5 µg/mL and 125.7 ± 6.0 µg/mL, respectively (Table 1). The lower the IC₅₀ value, the higher the antioxidant activity, as the decrease in absorbance of the DPPH or ABTS radicals correlates with the antioxidant potential of the sample [21]. As a positive control, ascorbic acid showed DPPH IC₅₀ of 9.4 ± 1.9 µg/mL and ABTS IC₅₀ of 71.8 ± 0.2 µg/mL, representing 34% (for DPPH assay) and 57% (for ABTS assay) of the IC₅₀ value of the extracts, respectively. In the previous literature, the reported DPPH IC₅₀ values and ABTS IC₅₀ values of *O. japonicus* extracts were 585–1942 µg/mL and 1135–2638 µg/mL, respectively [16]. In FRAP assay, the reducing power of the *O. fimbriata* extracts was determined to be 115.0 mmol/L, which was 30% of that of ascorbic acid (388.0 mmol/L) (Table 1). The high antioxidant activity of the *O. fimbriata* extracts is presumed to be due to the high TPC and TFC, particularly major flavonoids (epicatechin gallate, quercetin, and kaempferol) known as strong antioxidants.

Table 2 summarizes the extraction process to obtain antioxidants from *Orostachys* species [7,16,22–26]. To date, research on antioxidant characterization has focused on only *O. japonicus* among the *Orostachys* species. In general, the solid–liquid extraction (SLE) method was used to recover antioxidants from *O. japonicus*. The TPC and TFC of the *O. japonicus* extracts were 7.8–120 mg/g extract and 6.2–55.5 mg/g extract, respectively. However, most studies have focused on the quantification of the antioxidant content of the extracts, and few studies have been conducted on their activity. In this study, *O. fimbriata*

extracts were prepared by applying the ultrasonic-assisted SLE (USLE) method. USLE has been emphasized for extracting bioactive compounds due to its advantages such as high extraction efficiency, simplicity of operation, and less time dependency [22]. We extracted the antioxidants from *O. fimbriata* with a high yield by USLE, and we evaluated its antioxidant activity for the first time by in vitro test. In order to apply the *O. fimbriata* extracts in the pharmaceutical industry, it is necessary to identify major antioxidants for the extracts and evaluate their pharmacological effects through in vivo tests in the near future.

Table 2. Summary of the antioxidant content and activity of extracts from *Orostachys* species.

Species	Method	Extraction Conditions				Extract Type	Antioxidant Properties				Ref.
		Solvent	°C	h	g/L		TPC (mg/g)	TFC (mg/g)	DPPH IC ₅₀ (µg/mL)	ABTS IC ₅₀ (µg/mL)	
<i>O. japonicus</i>	SLE	MeOH (pure)	50	10	50	freeze-dried	7.8 (CAE)	-	-	-	[7]
<i>O. japonicus</i>	SLE	MeOH (85%)	95	3	100	freeze-dried	8.3 (CAE)	7.2 (QE)	-	-	[23]
<i>O. japonicus</i>	SLE	Water	95	3	100	dried	9.2 (CAE)	6.2 (QE)	-	-	[24]
<i>O. japonicus</i>	SLE	DW	70	2	100	dried	15.4 (CAE)	11.5 (QE)	1942	2638	[16]
<i>O. japonicus</i>	SLE	MeOH (70%)	RT	24	100	dried	23.8 (CAE)	15.8 (QE)	615	1313	[16]
<i>O. japonicus</i>	SLE	EtOH (70%)	RT	24	100	dried	24.1 (CAE)	16.0 (QE)	585	1135	[16]
<i>O. japonicus</i>	SLE	EtOH (70%)	RT	72	100	freeze-dried	120.0 (CAE)	17.0 (QE)	-	-	[25]
<i>O. japonicus</i>	SLE	Water	85	2	40	freeze-dried	16.0 (GAE)	16.4 (CE)	-	-	[26]
<i>O. japonicus</i>	SLE	EtOH (70%)	60	2	40	freeze-dried	60.0 (GAE)	55.5 (CE)	-	-	[26]
<i>O. fimbriata</i>	USLE	MeOH (pure)	45	0.25	-	freeze-dried	288.5 (GAE)	159.7 (RE)	28	126	This study

SLE, solid–liquid extraction; USLE, Ultrasonic-assisted SLE; RT, room temperature; TPC, total polyphenol content; TFC, total flavonoid content; CAE, caffeic acid equivalent; GAE, gallic acid equivalent; QE, quercetin equivalent; CE, catechin equivalent; RE, rutin equivalent.

3.2. Identification of Major Flavonoid in *Orostachys fimbriata* Extracts

The major flavonoids in *Orostachys* species such as epicatechin gallate, quercetin, and kaempferol were quantified by HPLC-DAD system (Figure 3). After that, the identification of flavonoids was performed by the HPLC-DAD-ESI-MS system. Epicatechin gallate, quercetin, and kaempferol in *O. fimbriata* extracts were detected at m/z 443, 303, and 287, respectively (Figure 4). This was consistent with the results of LC-MS analysis of the standard compound (Figure S1).

Quantitatively, 1 g of *O. fimbriata* extracts contained 34.5 mg, 10.6 mg, and 4.2 mg of epicatechin gallate, quercetin, and kaempferol, respectively (Table 3). A 159.7 mg of total flavonoids were present in 1 g extract (Table 1), of which 21.6% was proven to be epicatechin gallate. Quercetin and kaempferol accounted for 6.6% and 2.6% of the total flavonoids in the extracts, respectively. These results are consistent with the report of Lee et al. [3], who found that the major flavonoids of *Orostachys* spp. were epicatechin gallate (about 39% of the TFC), quercetin, and kaempferol.

The potential for utilization of resources can be evaluated based on the mass balance [27,28]. Figure 5 shows the mass balance in the overall process of recovering flavonoids from 1000 g (dry basis) of *O. fimbriata*. It is estimated that 83.8 g of the extract can be recovered through the USLE process. The extract contains 13.4 g of flavonoids, specifically epicatechin gallate, quercetin, and kaempferol, accounting for 2.9 g, 0.9 g, and 0.4 g, respectively. Several previous studies have identified and quantified these three flavonoids in medicinal herbs. Yeo et al. [29] reported that the epicatechin gallate, quercetin, and kaempferol contents of *Lycoris chejuensis* were 49 mg, 108 mg, and 38 mg per 1000 g biomass, respectively. In the case of the holly leaf, an herbal medicine, it contains 100 mg of kaempferol per 1000 g biomass [30]. Widely present in medicinal herbs, these flavonoids are responsible for their pharmacological effects. Epicatechin gallate, one of the catechins, possesses pharmacological functions such as antitumor, anti-inflammatory, and antidiabetic activities [31]. Quercetin as a powerful antioxidant can inhibit the oxidation of low-density lipoprotein and can reduce the risk of cancer [32]. Kaempferol is a strong lipophilic antioxidant, and an increased intake of it functions to be cardioprotective [33]. Therefore, *O. fimbriata* extracts, rich in these flavonoids, are expected to be used as nutraceutical and pharmaceutical materials due to their pharmacological functions and advantages for

human. This study is significant in that it identified major flavonoids as potential evidence that the unexplored *O. fimbriata* could be used as a traditional herbal medicine.

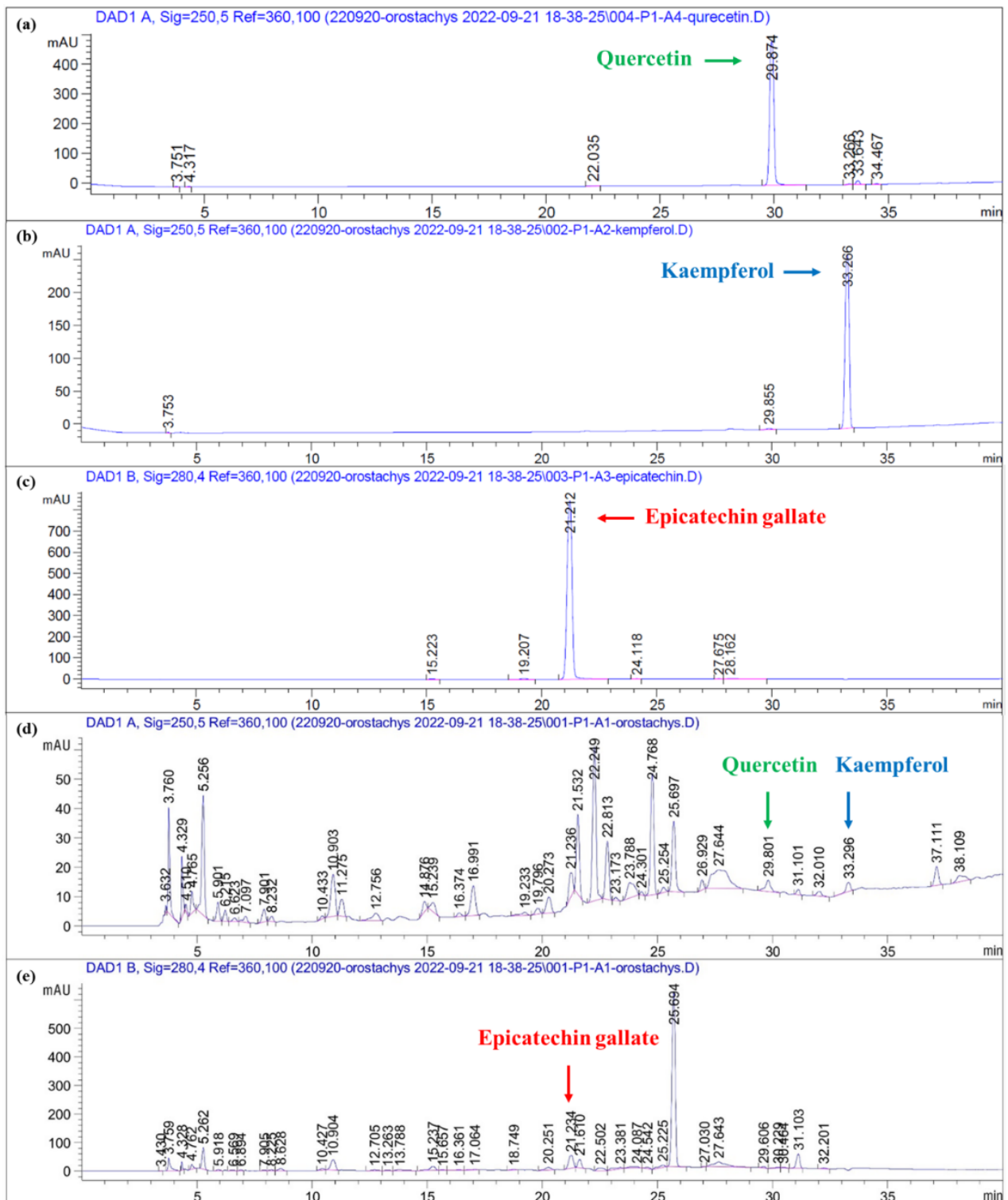


Figure 3. HPLC-DAD spectra for detection of quercetin standard (a), kaempferol standard (b), and epicatechin gallate standard (c), and these flavonoids in *O. fimbriata* extracts ((d), detected at 250 nm; (e), detected at 280 nm).

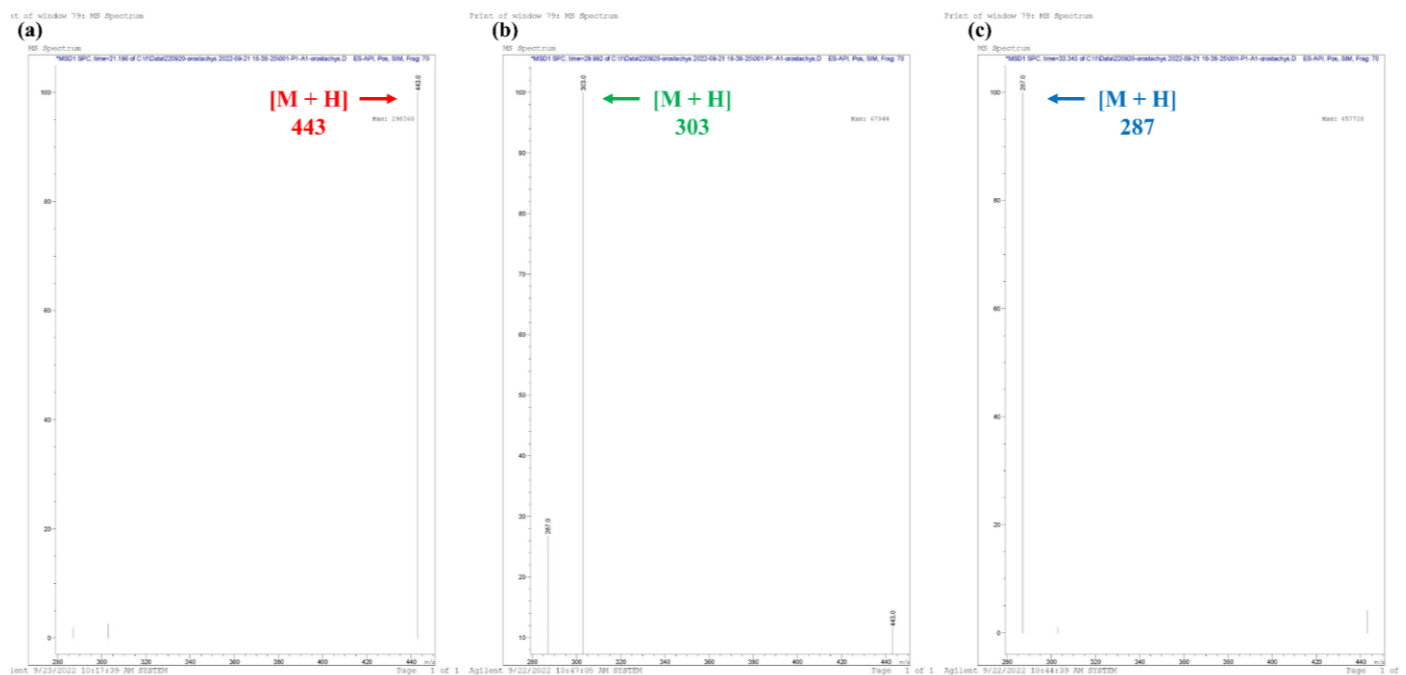


Figure 4. Mass spectra for identification of epicatechin gallate (a), quercetin (b), and kaempferol (c) in *O. fimbriata* extracts.

Table 3. Quantification of epicatechin gallate, quercetin, and kaempferol in *Orostachys fimbriata* extracts.

Compounds	Content in <i>O. fimbriata</i> Extracts (mg/g)
Epicatechin gallate	34.5
Quercetin	10.6
Kaempferol	4.2

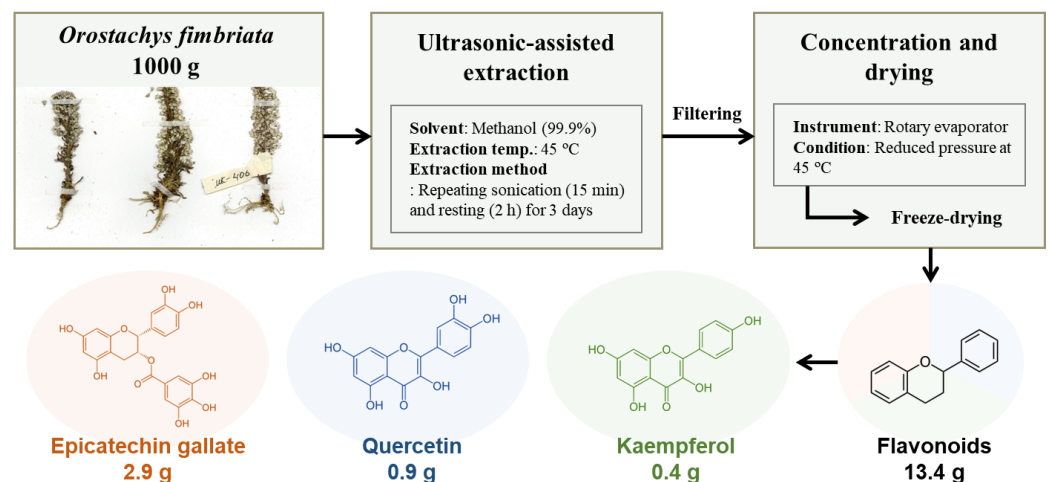


Figure 5. Mass balance of overall process for flavonoid recovery from 1000 g (dry basis) of *Orostachys fimbriata*.

4. Conclusions

In this study, for the first time, we attempted the antioxidant characterization of *O. fimbriata* extracts. The contents of polyphenols, flavonoids, epicatechin gallate, quercetin, and kaempferol in the prepared *O. fimbriata* extracts were quantified as 288.5 ± 7.4 mg, 159.7 ± 8.3 mg, 34.5 mg, 10.6 mg, and 4.2 mg per g of the extract. In addition, the *O.*

fimbriata extracts had powerful antioxidant activity compared to reported activities of *O. japonicus* extracts. Therefore, since *O. fimbriata* is expected to have pharmacological potential, related studies of not only *O. japonicus* but also *O. fimbriata* should be deepened. Based on the results of this study, we are planning a study to evaluate the in vivo bioactivity of *O. fimbriata* extracts.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae8111092/s1>, Figure S1: Mass spectra for identification of epicatechin gallate (a), quercetin (b), and kaempferol (c) standards.

Author Contributions: Conceptualization, J.L. and H.S.; methodology, K.H.L. and S.K.; validation, Y.C. and H.Y.Y.; formal analysis, J.L. and H.S.; investigation, K.H.L. and S.-Y.K.; resources, G.M. and S.-Y.K.; writing—original draft preparation, J.L. and H.S.; writing—review and editing, Y.C. and H.Y.Y.; visualization, J.L. and S.K.; supervision, Y.C. and H.Y.Y.; funding acquisition, Y.C. and H.Y.Y. All authors have read and agreed to the published version of the manuscript.

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